



Faculty of Resource Science and Technology

**EFFECT OF THE TEMPERATURE ON THE GROWTH OF
ENTEROCOCCUS FAECALIS CULTIVATED IN ALKALIPHILIC
CONDITIONS USING HYDROLYSED SAGO STARCH.**

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Effect of the temperature on the growth of *Enterococcus faecalis* cultivated in alkaliphilic

conditions using hydrolysed sago starch.

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Declaration

I hereby declare that the study entitled 'Effect of the temperature on the growth of *Enterococcus faecalis* cultivated in alkaliphilic conditions using hydrolysed sago starch' is my original work except for the citation on the sources.



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List of Abbreviations

LAB	Lactic acid bacteria
LAF	Lactic acid fermentation
NaOH	Sodium hydroxide
°C	Degree Celsius
HPLC	High Performance Liquid Chromatography
OD	Optical Density
HSS	Hydrolysed Sago Starch
HCL	Hydrochloric acid
DCW	Dry Cell Weight
CFU	Colony-forming unit
Rpm	Revolutions per minute

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Effect of the temperature on the growth of *Enterococcus faecalis* cultivated in alkaliphilic conditions using hydrolysed sago starch.

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Abstract

The objective of this study was to investigate the effect of the temperature on the growth of *Enterococcus faecalis* cultivated in alkaliphilic condition using hydrolysed sago starch. Batch fermentation was carried out in three different temperatures 37 °C, 42 °C and 47 °C with pH 8 in 1L fermentor under an agitation rate 200rpm and supplied by 100g/L of hydrolysed sago starch. The result showed that, *E.faecalis* can grow in the three different temperatures with the production of different amount of lactic acid it is was 114.77g/L at temperature 37 °C , follow by 42°C it is 102.58g/L and 47 °C is 65.76g/L. The best temperature for the growth and production of lactic acid in this study is 37 °C.

Keywords: *Enterococcus faecalis*, Hydrolysed sago starch (HSS), temperature, lactic acid, fermentation

Abstrak

Objektif kajian ini adalah untuk mengkaji kesan suhu ke atas ketumbesaran *Enterococcus faecalis* dalam keadaan alkaliphilic menggunakan kanji sagu terhidrolisi. Proses penapaian kumpulan telah dipilih dalam kajian ini. Langkah penapaian telah dijalankan dalam tiga suhu yang berbeza 37 °C, 42 °C dan 47 °C dengan pH 8 dalam fermentor 1L bawah 200rpm kadar pengadukan dan menggunakan 100g/L kanji sagu terhidrolisis. Hasil kajian ini menunjukkan bahawa, *E.faecalis* boleh hidup dalam tiga suhu yang berbeza dengan penghasilan jumlah acid laktik yang berbeza iaitu 114.77g/L pada suhu 37 °C, 102.58g/L pada 42°C dan 65.76g/L pada 47 °C. Suhu yang paling sesuai untuk ketumbesaran dan penghasilan acid laktik dalam experiment ini ialah 37 °C.

Kata kunci: *Enterococcus faecalis*, kanji sagu terhidrolisis, suhu, acid laktik, penapaian

1.0 Introduction

Enterococcus faecalis is one species of the Lactic acid bacteria (LAB) group. The classification of LAB is based on the morphology, mode of glucose fermentation, substrate spectrum, growth at different temperature, ability to grow at high salinity concentration, acid, alkaline or ethanol tolerance as well (Axelsson, 2004). LAB are gram positive, non-spore, forming rods, catalase negative, they do not reduce nitrate, non-producing and able to utilize glucose as carbon source. LAB is characterized by the production of lactic acid as major catabolic product, they have a requirement for complete growth factor and an unequivocal definition of LA is not possible (Axelsson, 2004).

There are two types of LAB it is homofermentative and heterofermentative. Homofermentative LAB is the bacteria that produce LA as the main product, while for heterofermentative they produce it has byproduct such as acetic acid and ethanol. There are numerous species of bacteria and fungi that are capable to produce relatively large amount of LA from carbohydrates (Atkinson & Mavituna, 1991). On the other hand, in the industrial fermentation lactobacillus species is more prefer because of it has higher conversion yield and rate of metabolism (Mercier & Yerushalmi, 1991).

LAB usually involves in the food and feed fermentation. Other than that, it is also use as preservation as well as food digestion in the intestinal tracts of humans and animal. Due to be tolerance again ethanol and acid, LAB can grow well under this condition. Besides, industrially used LAB properties suitable for synthesis of pure isomers and tolerance to high product con-

centration and high temperature and it is useful for the minimize contamination of the culture from other organism (Tsai *et al.*, 1993). According to Lonvaad-Funel *et al.*, (1988) LAB also metabolizes the three main acids of multi tartrate, malate and citrate.

Enterococcus is the genus of LAB of the phylum Firmicutes. Enterococci is common gram positive cocci that found in the bowel of human and other animals, and this microorganism was classified as streptococci on the basic of their morphology (Mushtaq Ahmad *et al.*,2002). However, DNA homology studied had demonstrated that members of the genus Enterococcus are closely related to each other but not a member of the genus streptococcus (Schleifer & Kilpper-Bälz, 1984; Collin *et al.*, 1984).

Enterococci are able to survive in a range of stresses and hostile environment. This species of LAB can stand high temperature. Temperature is the most important factor that affect bacterial growth rate. It affects the type and amount of the microbial growth. Microorganism is classified into three groups according to their temperature preferences such as psychrophilic, mesophilic and thermophilic organism. The optimum temperature is the best growth of the microorganism. Each 10°C increases will approximately double the chemical rate.

According to Van den Berghe *et al.*, (2006) Enterococcus species will grow at range of temperatures from 5 to 50 °C. Besides that, both of *E. faecalis* and *E. faecium* can survive at 60 °C for 30 minute, making *Enterococcus* species distinguishable from other closely related genera such as *Streptococcus* (Moreno *et al.*, 2006). Other than that both of the species can grow in a wide range of pH 4.6-9.9, with the optimum being 7.5 (Van den Berghe *et al.*, 2006).

Over the past few decades, studies were done on fermentation processes. In fermentation processes, bacteria or other microorganism produce LA as they metabolize carbon containing raw

material. Fermentation processes are characterized by biological degradation of metabolites such as LA, ethanol and citric acid (Maher *et al.*, 1995). Batch fermentation is use in this study. The batch fermentation is a closed system and nothing is added apart from oxygen.

This study will be carried out with three different temperatures, 37 °C, 42 °C and 47 °C. The problem statement of this study is the *E. faecalis* might not be able to stand the effect of high temperature, the high pH, low productivity from batch fermentation, contamination from the external microorganism and expensive raw material.

The objective of this study was to determine the ability of *E. faecalis* to growth in alkaliphilic condition at pH 8 and to investigate the effect of temperature 37 °C, 42 °C and 47°C on the growth of it using HSS. It is also to determine the production rate of LA between the three different temperatures The result showed that, *E. faecalis* can grow in the three different temperatures with the production of different amount of lactic acid it is was 114.77g/L at temperature 37 °C , follow by 42°C it is 102.58g/L ,42°C it is 65.76g. The best temperature for the growth and the production of LA in this study is at 37 °C.

2.0 Literature Review

2.1 Characteristic of *Enterococcus faecalis*

Enterococci are organisms with a remarkable ability to adapt to the environment and acquire antibiotic resistance determinants. It belongs to a group of organisms known as LAB that produce bacteriocins. It is a common cause of nosocomial infection as aetiological agents of hospital-associated infections in US hospitals, with *E. faecalis* and *E. faecium* currently isolated in an approximately 1.5:1 ratio (Arias *et al.*, 2010). Enterococci are found in the feces of most healthy adults, and the number of *E. faecalis* can be found more than *E. faecium* in the feces.

The most important variable of the growth conditions for *Enterococcus* species is pH, temperature and salt concentration (Gardin *et al.*, 2001). During the lag phase, temperature is the most important factor that influences growth, with stationary phase cells being the most resistant to heat (Gardin *et al.*, 2001).

E. faecalis is a gram positive, facultative anaerobic microbe that ferments glucose without gas production, it is an anaerobic organism that is ovoid in shape and may appear on smear in short chains, in pairs or as single cells. Most strains of *Enterococcus* genus possess the characteristic that has the ability to grow in 6.5% NaCl and at pH 9.6, grow at 10 and usually 45 °C, and it is also survive at 60 °C for 30 minutes (Sherman, 1937). It has a cation homeostasis which is thought to contribute to its resistance to pH, salt, metals and desiccation.

2.2 Sago starch

Sarawak is one of the sources of sago palm in Malaysia. The scientific name for sago is *Metroxylon sagu*. It is obtained from various palm stem especially from sago spongy center or pith. Starch is the mostly produced carbohydrate by plants and is the major reserve polysaccharides of green plants (Morrison & Karkalas, 1990).

Starch is a primary food component and a complex biodegradable carbohydrate that made up from thousands of glucose units (Zhou *et al.*, 2009). It is has primarily linear and branched chain of glucose molecules namely amylose and amylopectin (Fazilah *et al.*, 2011). Starch granules synthesized in amyloplasts and are deposited in the form of tiny granules in the major depots of seeds, tubers and roots. The granules of starch insoluble in water and resistant to many enzymes and chemicals action, by heating in water will disrupt the granular structure (Wang *et al.*, 1995).

Sago flour process can be characterized as a process industry because it transforms a raw material into a product such as sago flour that has many advantages to customer (Siti Mazlina *et al.*, 2007). The processing of sago palm to produce sago flour included mechanism such as maceration, crushing, kneading the pith, sieving, settling, washing and filtration.

Sago starch can be used as a staple food by Melanau in the third division of Sarawak making keropok (Shrimp crackers) (Sidaway & Balasingam, 1971), and tebaloi biscuits.

2.3 Batch fermentation

A batch culture operation is characterized by no addition to and withdrawal from the culture of biomass, fresh nutrient, and culture broth, it is initiated by addition of an amount of a cell culture to a sterile nutrient medium (Ali *et al.*, 2003). Batch fermentation can be defined as the process begins with inoculations and end with retrieval of the product happens inside a single fermentor with no intermediate steps.

A typical batch culture operation is strictly not a batch operation since it may involve addition of an acid or base for pH control and antifoam to suppress foaming in the culture and withdrawal of small portions of culture for assessing the status of the culture (Ali *et al.*, 2003). There are four typical phases of the growth, lag phase, log phase, stationary phase and death phase.

Advantages of batch fermentation is the risk of contamination or cell mutated is reduced as the growth period is shorter and low maintenance and low capital investment compared to other fermentation process.

2.4 Lactic acid

Lactic acid (2-hydroxypropanoic acid) is an invaluable chemical that was first discovered by the Swedish chemist Scheele in 1780, which isolated the lactic acid from sour milk (Sheeladevi *et al.*, 2011). It is an organic acid that been used in the food, cosmetic, pharmaceutical and chemical industries.

Three carbon organic acids, one terminal carbon atom is part of an acid or carbonyl group, the other terminal carbon atom is a part of a methyl or hydrocarbon group and a central carbon atom having an alcohol carbon group is a structure of lactic acid (Niju *et al.*, 2004). It has two optically active isomeric forms such as L (+) lactic acid and D (-) lactic acid. It is soluble in water and water miscible organic solvents but insoluble in other organic solvents, it is also exhibits low volatility.

Lactic acid contributed many advantages such as in food, pharmaceutical, leather and textile industries. Nowadays, LA is considered to be the one of the most useful chemicals used in the food flavoring, preservative and pharmaceutical industries. Besides that the consumption of it has increased because of its role as a monomer in the production of biodegradable PLA (polylactic acid) which is known as a sustainable bioplastic material (Young Jung *et al.*, 2006).

2.5 Temperature and alkaliphilic condition

The heat resistance of the enterococci has been known for a long time and this characteristic has been used to classify these microorganisms (Perez *et al.*, 1982). Heat resistance of enterococci depends on several factors such as age of cultures, external pH (White, 1953), and composition of the suspending medium (Perez *et al.*, 1982). The heat tolerance of bacteria could be affected by factors such as temperature of the medium in which bacteria are grown, age of the bacterial culture and rate of heating temperature (Sorqvist, 1994).

Temperature is one of the important factor that affect the growth of bacteria, various researchers have studied the effect of the temperature on LA production and they found out the optimal temperature between 41-45°C (Hofvendahl & Hagerdal, 2000). At temperature between 5-50 °C *Enterococcus* species able to growth but the LA production will decrease when the temperature is above 45°C. Goksungur & Guvenc (1997) study that the optimal temperature is at 45°C and it can be because of different substrates that being used during the fermentation process. Besides, there was a study that was carried out on the pH on LA production and it is conclude that the optimum pH was between 5-7 (Hofvendahl & Hagerdal, 2000 ; Goksungur & Guvenc, 1997).

Besides that, alkaliphilic conditions also effect the growth of the bacteria. Alkaline condition is the condition with high pH. Organisms with pH optima for growth in excess of pH 8, usually between 9 and 10 are defined as alkaliphiles (Desai *et al.*, 2004). The term alkaliphiles is usually refer to microorganisms that need alkaline media or condition for growth beside microorganism that grow in alkaliphilic condition experience more neutral values than the average value of their ecosystem owing to the nature of their microenvironment (Digrak, 2002).

3.0 Material and Method

3.1 Microorganism and preparation of Stock culture

In this study *E. faecalis* was used. The bacteria sample is provided by the Biochemistry laboratory of Faculty of Resources Science and Technology, UNIMAS. For the preparation of stock culture, 200ml media preparation was made using 20g/L glucose and 5g/L yeast extract. Mix the mixture of glucose and yeast extract until dissolved and then pour into universal bottle. Then autoclave it for 121°C for 20 minute and allowed to cool in room temperature. 20 ml of *E. faecalis* added into each of the universal bottle. The incubating condition for the media is 24 hours at 37 °C, then stored in refrigerator and maintained in sterile condition.

3.2 Preparation of inoculums

20g/L glucose and 5g/L yeast extract was used to make inoculums. 20g/L glucose and 5g/L yeast extract weight and dissolved into 200ml of distilled water and pour in blue cap bottle and autoclave for 121°C for 20 minute. 5g/L of yeast extract was weight and then dissolved in 100ml of distilled water and autoclave. After that, the stock culture taken out and incubate in incubator for 3 hours to multiply the cell. The supernatant discharged and the pellet cell was mix with culture media for the inoculums and incubated for 24 hours at 37 °C. After the incubation time, it was centrifuge for 10 minute at 8000rpm. Discharge supernatant and a little amount of sterile distilled water added to dilute the pellet and were added to fermentor using a small funnel or add it to new

medium to subculture or giving the bacteria new environment to prevent it from stop growing. Subculture was done every one day to make sure the bacteria continued growing.

3.3 Preparation of hydrolysed sago starch

HSS used as the main source for the glucose. It was made by two methods known as liquefaction and saccharification process. 130g of Sago flour was weight and dissolved in 1000ml of tap water. The sago starch converted into glucose by enzymatic hydrolysis by double boiling method. The pH of the suspension was adjusted to 6.5 by the addition of acid or base. Termamyl (0.5µl/g) was added into the suspension at temperature of 90 °C and the process took 2 hours to complete. The liquefied sago starch was cooled down. Then the pH was checked and adjusted to 4.5. After that, Dextrozyme (0.5µl/g) was added at temperature of 60 °C and the process took 24 hours. Sago starch contains 15% moisture, the wet weight of sago starch must be weight to obtain dry weight by following equation:

$$Wet\ weight = \frac{Dry\ weight}{0.85}$$

3.4 Fermentation process

1000ml of working volume was used in this fermentation process. The concentration of HSS used was 100 to 130g/L. The fermentation process operated for 48 hours. The sampling process was done at 0 hours, 4 hours, 6 hours, 8 hours, 12 hours 18hours and 24 hours until the glucose fully consumed by the bacteria and then the fermentation process was stop. During the fermentation 10mL of the broth sample was taken out to determine the LA and residual glucose concentration. Besides, the microbial growth was measured using spectrophotometer to study the growth of the bacteria on every sampling hour.

3.5 Analysis

3.5.1 Glucose determination

The analysis of glucose concentration was analyzed using biosensor glucose analyzers. 2ml of sample added into 100ml volumetric flask. Then the mixtures were injected to biosensor analyzer. The reading of the analysis was recorded.

3.52 Dry cell weight (DCW)

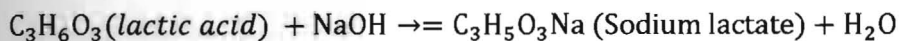
DCW measurement done by sampling 10ml of liquid culture, centrifuging the sample, discarding the supernatant, and resuspending the sample (Stone,1992). Cell separation technique was done using centrifugation or filtration. The sample washed with water or buffer and dried typically at 80 °C for 24 hours or at 110 °C for 8 hours. After washing, the cell dried was weight using analytical balance. The dry weight of the residual solids calculated at the difference between the weight of the filter before and after use. Cell dry weight will be determined as below,

$$DCW \left(\frac{g}{l} \right) = \frac{(\text{wt dried filter} + \text{cell, g}) - (\text{wt filter, g})}{\text{Sample volume}} \times 10^3$$

3.53 Lactic acid determination

The determination of lactic acid calculated using the weight of NaOH consumed during its titration in situ. The conversion factor of 0.69 was used to report the concentration of lactic acid. The chemical equations below show the relationship between lactic acid and NaOH and the way that the 0.69 factor was determined.

From the calculation of the LA conversion factor from NaOH, the factor of 0.69 was used to know theoretically the production of LA using the weight of 10M NaOH that was pumped in during the fermentation period. The weight of NaOH was multiply with LA conversion factor to obtain the concentration of LA that was produced.



Conversion factor of 0.69 was obtained by:

From the chemical equation of LA and NaOH:

Molar mass: NaOH = 40g/mol, LA= 90 g/mol

Ration: NaOH (1): LA (2.25)

$$1\text{M} = 40 \frac{\text{g}}{\text{mol}}, \quad 10\text{M} = 400 \frac{\text{g}}{\text{L}} = 0.4 \frac{\text{g}}{\text{mL}} \quad \text{density} = 1.3 \frac{\text{g}}{\text{mL}}$$

Mass per mL of 10M NaOH solution

$$\rho = \frac{\text{Mass}}{\text{Vol}}, \quad \text{Vol} = \frac{M}{\rho} = \frac{1 \text{ g}}{1.3 \frac{\text{g}}{\text{mL}}} = 0.769 \text{ mL} \quad \text{density} = 1.3 \frac{\text{g}}{\text{mL}}$$

Therefore:

1 g of 10 M NaOH contain

$$g_{\text{NaOH}} = 0.4 \frac{\text{g}}{\text{mL}} \times 0.769 \text{ mL} = 0.3077 \text{ g}$$

Equivalent of Lactic acid in grams

$$\text{Lactic acid (g)} = \frac{90 \frac{\text{g}}{\text{mol}}}{40 \frac{\text{g}}{\text{mol}}} \times g_{\text{NaOH}} = 2.25 \times 0.3077 = 0.69$$

3.54 Colony-Forming unit (CFU)

Agar prepared using lactobacillus selective agar 48g/L. 1ml of broth sample that collected from the fermentor use to make dilution for CFU. The dilution was done from $\times 10^1$ to $\times 10^7$. 900 μ l of sterilize distilled water was pipette to every 7 eppendorf tube, and then vortex the tube to mix the solution and dilution of $\times 10^6$ and $\times 10^7$ used. Three of Petri dish was use for each of the dilution. Using pipette drop it on Petri dish, spread well until dry on the agar surface and incubated it for 24 hours. CFU done in this study to show the growth of the bacteria from the 0 hour and 24hour after the fermentation was started.

3.5.5 Determination Optical Density (OD) of sample

Optical density was obtained by diluting the sterile sample using distilled water. The reading of OD with cell and without cell was taken. The OD of the sample was analyzed by using Spectrophotometer at wavelength set at 590nm.

4.0 Results and Discussion

4.1 The NaOH consumption at temperatures 37 °C, 42 °C and 47 °C

Table 1, 2 and 3 shows the consumption of NaOH. Then by stoichiometric of the action the weight of NaOH can be converted by using factor 0.69 to LA. The following graph shows the LA production against time and the rates of LA production in each hour. The production of LA increases as the time increase. The assume weight of NaOH used in this fermentation is 189g/L. The concentration of HSS for the nutrient supply provided was between 100g/L and 130g/L for the theoretical values. In this study, it is show that *E. faecalis* was able to produce LA at this three different temperature eventhought the production of LA was has a significant different amount. This might be caused by the external contamination, temperature and the pH used during the experiment. The fermentation process was triplicate under the same condition to get constant result.

While in the graph for the LA rate production per hour show that the productions of the LA was decreased. This is may be caused by the low number of the cell in the broth during the process. Besides that, the increase number of cell in the broth will make the bacteria compete for the nutrient. During this study, the growth of this bacteria was study, and it show that it is slow to multiple and growth if the parameter is not control. Other than that this bacteria must adapt itself into the fermentation media to multiple and reproduce rapidly. For a low number of the cell that cultivate in 100g/L of HSS it take a long time to finish the glucose. So in this experiment, the fermentation process was conducted in two days for each repeat, to observe the growth of the bacteria in a fixed day.